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Exploring the rumen microbial community in Guizhou White goats at different ages

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ABSTRACT - This study evaluated the changes in rumen microbiome during the process of age development of farming Guizhou White goats from Southwest China. We conducted high-throughput 16S rRNA gene sequencing to investigate the diversity, structure, and composition of goat rumen microbiota (RM) of 21 goats of different age groups (1, 6, and 12 months). We found that volatile fatty acids (i.e., acetate, propionate, and butyrate) fermented by microbes were found to increase significantly in the six- and one-month-old goats. Results of the genera abundance analysis showed that abundance of eight and seven taxa decreased in six- and one-month-old goats, respectively, compared with that in 12-month-old goats. Additionally, differences in six taxa in six-month-old goats and in one taxon in one-month-old goats were found. In addition, specific gut microbiome was found, which was significantly correlated with rumen fermentation parameters in Guizhou White goats. These results revealed the signature microbiota in RM during various developmental stages in goats raised in Southwest China and can also provide a guiding tool for evaluating rumen health of ruminants worldwide.

Keywords: age development, dynamics distribution, fermentation, rumen microorganisms

1. Introduction

The rumen is the site of digestion of plant fibers, which are ultimately converted into chemical compounds and assimilated by the animal (Mackie, 2002; Zou et al., 2020). This process is mainly mediated by microbial degradation and fermentation. Therefore, it is important to study the changes in the microbiome of the rumen during age development. In the pre-weaning stage, the rumen is not active in digestion of plant materials. As the animal ages, the structure and physiological characteristics of the rumen alter with the accumulation of a variety of microorganisms. Rumen microorganisms produce different fermentation products that greatly aid nutrient absorption and the development of rumen wall villi (Beharka et al., 1998; Bretschger et al., 2010; Wallace, 1992). Both the diet and age of animals significantly affect the bacterial population (Jami et al., 2013; Jiao et al., 2015a; Li et al., 2019; Zhang et al., 2019). During the first two years of life, cows show dynamic changes in the bacterial composition of rumen content, which further leads to an age-related rise in

Bacteroidetes and Firmicutes phyla and a reduction in Proteobacteria (Li et al., 2019). Three phases have been reported in goats: microbial colonization of rumen contents, functional attainment, and anatomic growth, occurring at one, two, two+ months, respectively (Jiao et al., 2015b).

At present, high-throughput sequencing-based technologies have been effectively applied for the investigation of complex bacterial ecosystems, such as gut microbiota (Glenn, 2011; Wang et al., 2018). The Guizhou White goat is a native breed raised for meat; it is popular due to its suitability for grazing in mountainous areas, and has many other favorable traits, including body size, high fertility, pleasant taste, and remarkable skin quality (Sui et al., 2015). Guizhou White goats have been prevalent in Southwest China for a long period because of their excellent production performance, which is related to both genetic factors and their associated microorganisms. Despite this, the relationship between age and composition of the rumen microbiota (RM) in goats, particularly in Guizhou White goats, has rarely been studied.

Therefore, a high-throughput sequencing-based method was conducted in the current study to investigate the microbial community of rumen samples from goats of various ages (from one month to 12 months). Discovery of differences in both the composition and percentage of bacterial populations will explain the excellent production performance of Guizhou White goats.

2. Material and Methods

This study was carried out in Yanhe, Guizhou, China (28°58'83" N latitude, 108°19'80" E longitude, and elevation of 968 m). Research on animals was conducted according to the institutional committee on animal use (case number: 20190312). Animals were maintained and processed in accordance with the institute's guidelines for the Care and Use of animals.

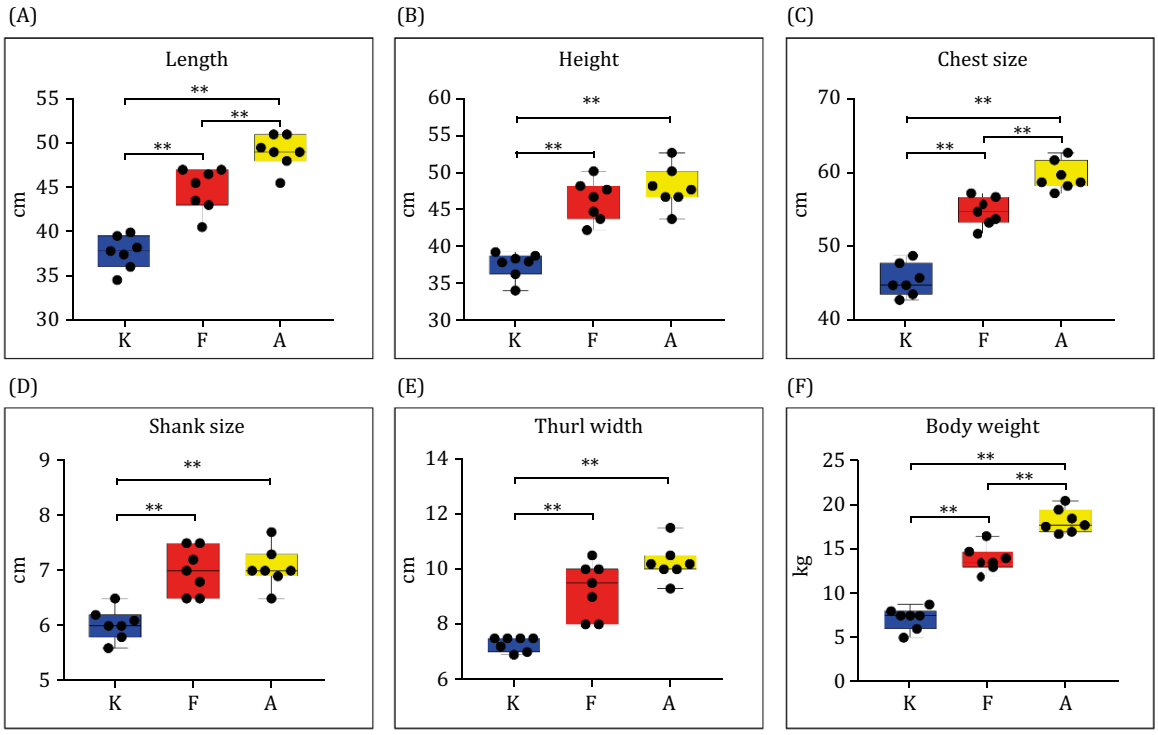
2.1. Goats and sample preparation

A total of 21 Guizhou White goats of various ages [seven kids (K) aged one month; seven fattening goats (F) aged six months; and seven adult goats (A) aged 12 months] were obtained from a commercial farm in Southwest China (Guizhou Province) and used in this experiment. Dietary composition and growth performance were detailed in Table 1 and Figure 1, respectively. None of goats selected for the experiment had any diseases prior to sample collection. After slaughter, fluid samples (approximately 10 mL) from each animal's rumen were obtained after filtration by using four layers of sterile gauze. Following centrifugation at 1000 × *g* for 15 min at 4 °C, rumen contents were acquired (Lv et al., 2019). The resultant pellet was put into 2-mL tubes and quickly exposed to liquid nitrogen vapors. All samples (of seven goat kids abbreviated as K1 through K7; seven fattening goats abbreviated as F1 through F7; and seven adult goats abbreviated as A1 through A7) were transported to the laboratory and later stored at −80 °C until the experimental analysis.

Table 1 - Dietary compositions

Sample	Item (%)								
	Crude protein	Moisture content	Crude ash	Calcium	Total phosphorus	Crude fat	Crude fiber	Neutral detergent fiber	Acid detergent fiber
Concentrate	18.66	12.94	7.00	1.30	0.66	2.16	6.34	24.32	6.61
Grass fodder	12.58	50.06	9.41	1.31	0.21	1.88	9.51	16.69	10.06

Feeding and management were conducted by semi-intensive grazing in our study, i.e., goats fed on grass land with supplement of concentrate. Grass fodders were collected from five points of the grass land for analysis.



Groups: K - kids; F - fattening goats; A - adult goats.
Statistical significance was accepted at P<0.05.
** P<0.01.

Figure 1 - Growth performance of goats at different ages.

2.2. Chemical analysis

Ruminal content pH was measured immediately using a pH electrode (PB-10; Sartorius, Goettingen, Germany). Determination of rumen fluid ammonia nitrogen (NH_3-N) concentration by a phenol-sodium hypochlorite colorimetric method was performed after the liquid was thawed at 4 °C (Lv et al., 2019). Volatile fatty acids (VFA) concentration in the rumen fluid was quantified by gas chromatography (Jiao et al., 2014) using methyl valerate as the internal standard in an Agilent 6890 series GC equipped with a capillary column (HP-FFAP19095F-123, 30 m, 0.53 mm diameter and 1 mm thickness, Agilent Technologies, Santa Clara, CA, USA).

2.3. DNA extraction and 16S rRNA amplification

Bacterial DNA extraction was carried out through an Omega E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. Agarose gel electrophoresis 0.8% (w/v) was used for the evaluation of the quality of the extracted DNA, and a UV microspectrophotometer (NanoDrop 2000, USA) was used for evaluating the quantity.

Next-generation sequencing library constructions and Illumina MiSeq sequencing were carried out at BIOMARKER Technologies (Beijing, China). Briefly, the RM was characterized on the basis of the V3 and V4 hypervariable region of the 16S rRNA gene, which was amplified by bar-coded fusion primers (forward 5'-CCTACGGRRBGCASCAGKVRVGAAT-3 and reverse 5'-GGACTACNVGGGTWTCTAATCC-3'). Polymerase chain reaction (PCR) conditions were as follows: 94 °C for 5 min; 27 cycles at 94 °C for 30 s, 55 °C for 30 s, and an extension at 72 °C for 5 min. The PCR products were loaded on a 1.2% (w/v) agarose gel and visualized by gel-red staining. The expected PCR products were separated by gels and purified using a DNA Gel Extraction Kit (Axygen, USA) following manufacturer's instructions.

2.4. Illumina sequencing and data analysis

Purified PCR products were then subjected to next-generation sequencing (Illumina MiSeq platform) using a DNA library preparation kit (Illumina, USA) following manufacturer's instructions. Sequences of V3 and V4 were processed, spliced, and analyzed by BMKCloud (https://international.biocloud.net/).

The Illumina Miseq platform was used for sequencing the 16S rRNA gene from bacterial V3 and V4 regions. The data analysis package QIIME 2 (Quantitative Insights into Microbial Ecology (Caporaso et al., 2010) was used for evaluating the 16S rRNA-derived sequence data. Mothur (version v.1.30), (Schloss et al., 2009) was used for filtering out partial 16S rRNA sequences by considering mean quality score ≥20 bp and length ≥ 250 bp. Sequences were then allocated to each sample through precise matching with 10 bp barcodes. Next, the Uchime algorithm was employed for the detection of chimeric sequences through the Usearch tool containing a chimera-free reference database (Edgar et al., 2011). The sequence analysis, comprising operational taxonomic unit (OTU, at the 97% similarity level) clustering and taxonomic assignment, was carried out in Mothur (Schloss et al., 2009). The rarefying of the sequences was conducted before calculating the alpha and beta diversity statistics. QIIME was used for calculation of α diversity indices from rarefied samples employing richness and diversity indices of the bacterial community (i.e., Shannon, Simpson, Chao1, and ACE). Principal coordinate analysis (PCoA) was carried out by unweighted UniFrac. The differences among groups were evaluated by one-way analysis of similarity (ANOSIM).

2.5. Data analysis

Wilcoxon signed-rank test was performed to evaluate the differences in the α diversity, the variance of relative abundance among bacterial taxa, and the total copy number of bacterial 16S rDNA gene using the BMK Cloud platform (https://international.biocloud.net/). One-way analysis of variance (ANOVA) or Students' t test was conducted for the analysis of the difference of growth performance and rumen fermentation parameters. Data were analyzed as the mean ± standard error of mean (mean±SE), and $P \leq 0.05$ was used as the significance level. Linear discriminant analysis coupled with effect size (LEfSe) at the genus level was conducted to discover the bio-markers, and only scores that reached the linear discriminant analysis threshold >3.0 were included.

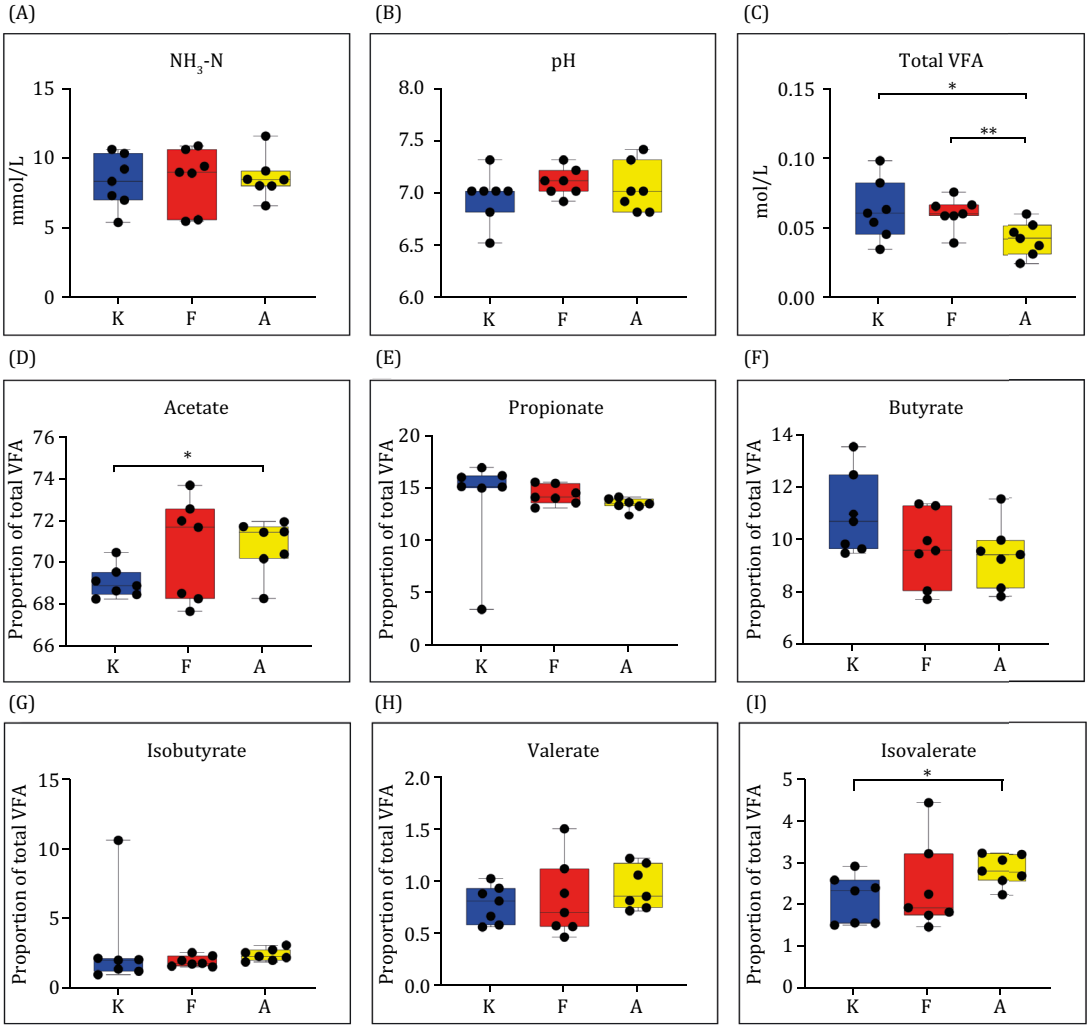
3. Results

3.1. Rumen fermentation parameters

Rumen fermentation parameters affected by the different ages were observed in this study (Figure 2). No difference among groups K, F, and A were found in the concentration of NH_3-N ($P>0.05$), and the same patterns of ruminal pH values were found (Figures 2A and 2B). Lower concentrations of total VFA in group A compared with groups F and K were observed ($P<0.05$) (Figure 2C). Results of analysis by the proportion of the total VFA showed that higher concentrations of acetate and isovalerate in group A compared with group K were observed ($P<0.05$) (Figures 2D and 2I). However, there was no difference in propionate, butyrate, isobutyrate, and valerate among the three groups ($P>0.05$) (Figures 2E-H).

3.2. DNA sequence data and microbial diversity in the rumen microbiome

A total of 228,418 pairs of readings were attained in this study; and 76,622, 76,545, and 75,250 raw readings were obtained from groups K, F, and A, respectively. After optimization of the original data, 216,013 effective sequences were attained. Based on the 97% OTU similarity, 72,636, 72,423, and 70,954 OTU were obtained from groups K, F, and A, respectively (Table 2). A total of 1,047 OTU were obtained from 21 goats, of which 995 coexisted among all groups considered core OTU (Figure 3).



Groups: K - kids; F - fattening goats; A - adult goats.
VFA - volatile fatty acids.
Comparison of rumen NH_3-N (A) and pH values (B). Significant differences of total VFA (C), acetate (D), propionate (E), and butyrate (F) were found. Differences of isobutyrate (G), valerate (H), and isovalerate (I). An ANOVA test was used for significance calculation after detection of homogeneity of variance.
Statistical significance was accepted at $P<0.05$.
* $P<0.05$; ** $P<0.01$.

Figure 2 - Differences on rumen fermentation parameters in goats.

Table 2 - Sequence data of samples

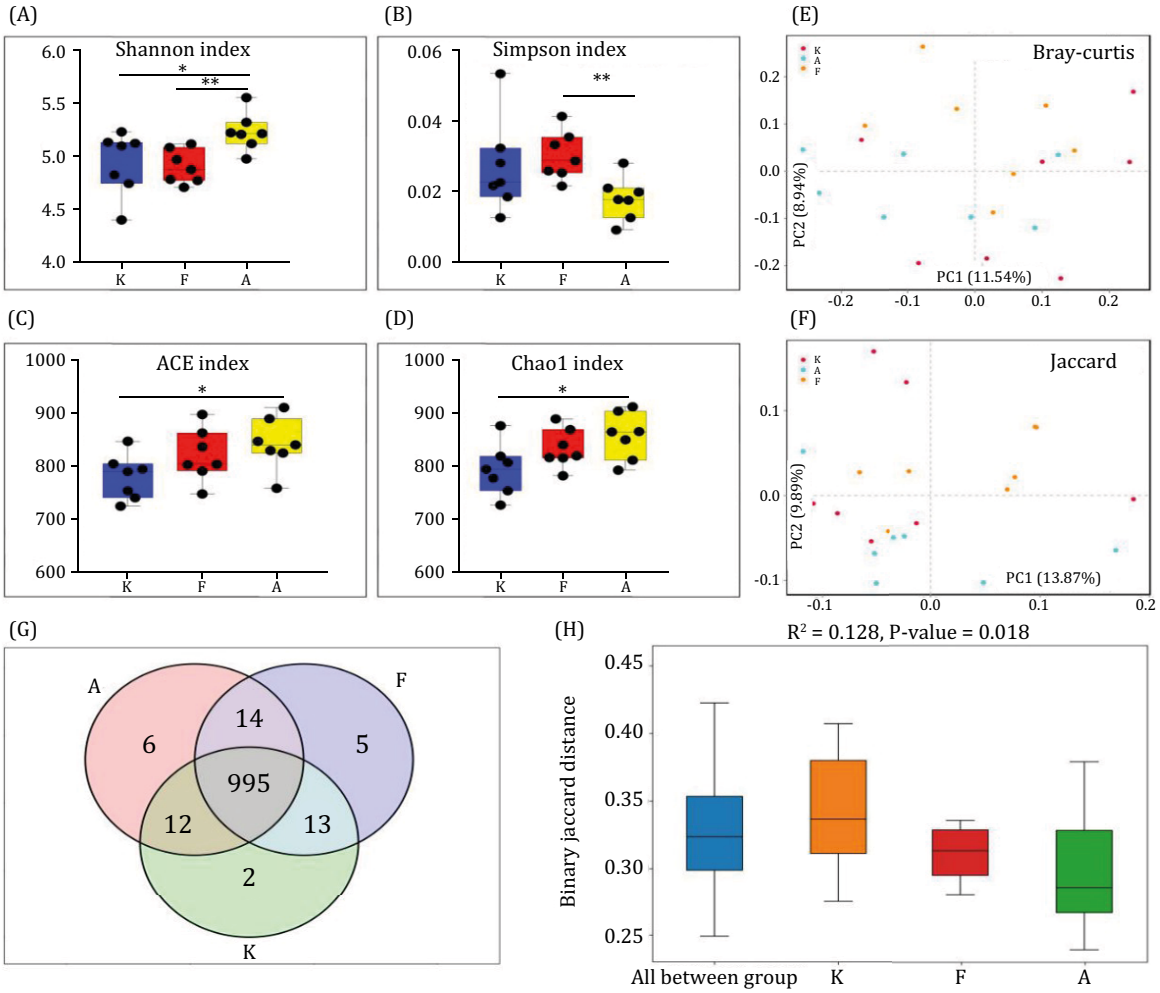
Sample	Raw read	Effective sequence	OTU
K1	80042	68537	735
K2	80002	70606	746
K3	80224	71144	827
K4	78906	71137	665
K5	80070	71243	757
K6	80107	71037	744
K7	79830	71811	708
F1	79989	70217	759
F2	79887	69026	733
F3	80125	70845	801
F4	80117	70969	825

Continues...

Table 2 (Continued)

Sample	Raw read	Effective sequence	OTU
F5	80021	69458	780
F6	79983	70569	718
F7	79975	70560	749
A1	79828	69558	789
A2	79725	70719	857
A3	79930	67372	831
A4	80189	69391	729
A5	79956	69570	743
A6	73451	63154	789
A7	79887	69212	807

OTU - operational taxonomic unit.



Groups: K - kids; F - fattening goats; A - adult goats.
A: Shannon index; B: Simpson index; C: ACE index; D: Chao1 index; E and F: PCoA analysis by using Bray-curtis and Jaccard distance, respectively; G: Venn diagram; H: ANOSIM analysis.
Points of different colors represent different groups. The distance between the two points represents the difference of RM. The numbers in the figure represent the unique or common operational taxonomic unit in each group.
Statistical significance was accepted at $P<0.05$.
* $P<0.05$; ** $P<0.01$.

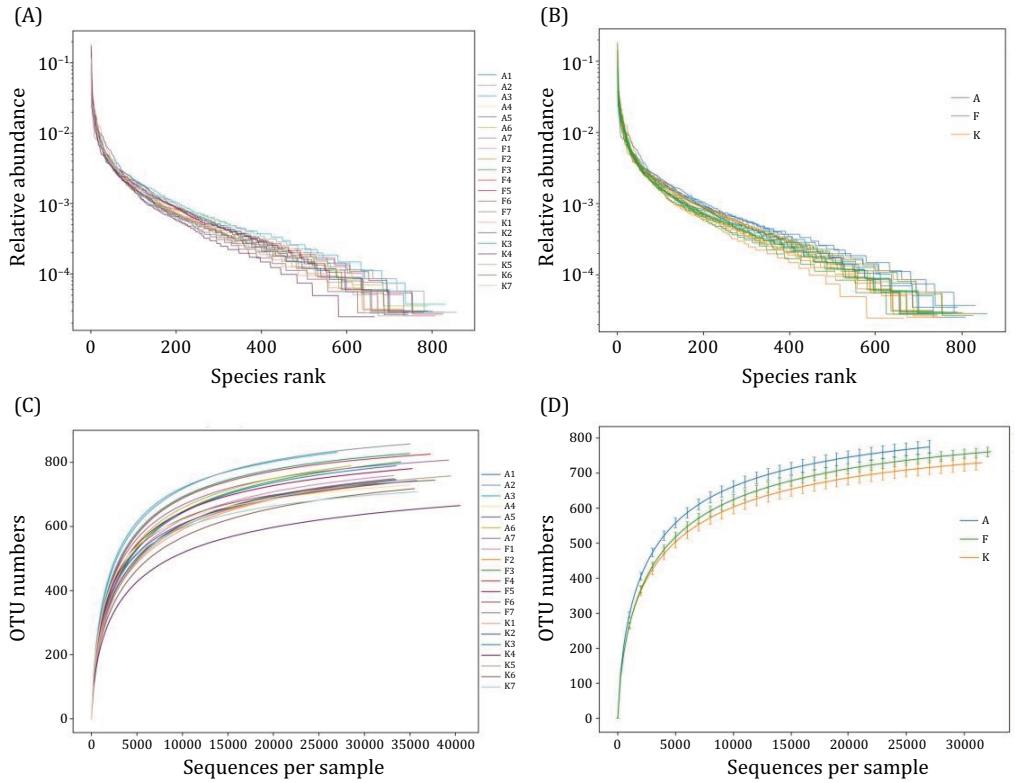
Figure 3 - Analysis of DNA sequence data, microbial diversity index, and bacterial community structures.

The core OTU included about 95% of the total OTU. Moreover, 2, 5 and 6 OTU were uniquely identified in groups K, F, and A, respectively (Figure 3G).

Multiple α-diversity indices were obtained to analyze the richness and diversity of the microbial community of the RM. In accordance with the Chao1 estimator, 789, 829, and 853 average OTU have been shown in samples from groups K, F, and A, respectively (Figure 3D), while the ACE index illustrated 790, 830, and 854, respectively (Figure 3C), which indicated the abundance of microflora of the RM in goats. The Shannon-Wiener index is a measure of the heterogeneity of a community. The Shannon indices of groups K, F, and A were 4.93, 4.89, and 5.22, respectively (Figure 3A). Intergroup analysis of Chao1 and the Shannon index illustrated that the abundance and diversity of the RM in group A were higher than that in groups K and F, whereas the difference between groups K and F was insignificant (Figure 3). A consistently lower Simpson index was observed in group A (Figure 3B). In addition, Good's coverage estimates for groups K, F, and A were 99.66, 99.62, and 99.66%, respectively, and all groups showed good coverage rate.

The PCoA of the Bray-Curtis and Jaccard distance matrix intuitively indicated the differences among all individuals or groups. The microbiota in all groups were not clustered well along the principal coordinates (Figures 3E and 3F). Moreover, ANOSIM revealed that the difference between groups was smaller than that within groups ($R = 0.128$, $P<0.05$), (Figure 3H).

The rank-abundance curve can indicate the abundance and evenness of species. Results showed that the OTU ranks were within 800, indicating that species composition of each sample was moderately rich. Curves in all samples were relatively flat, indicating that all the species compositions were comparatively homogeneous (Figures 4A and 4B). Furthermore, the curve plateaued at 30,000 effective sequences (Figures 4C and 4D).



OTU - operational taxonomic unit.
Groups: K - kids; F - fattening goats; A - adult goats.
Each curve represents a sample. A and B: rank-abundance curves; C and D: rarefaction curves describe effects of sequences on the number of OTU identified.
Abscissa: the species abundance order; ordinate: relative abundance of the species.

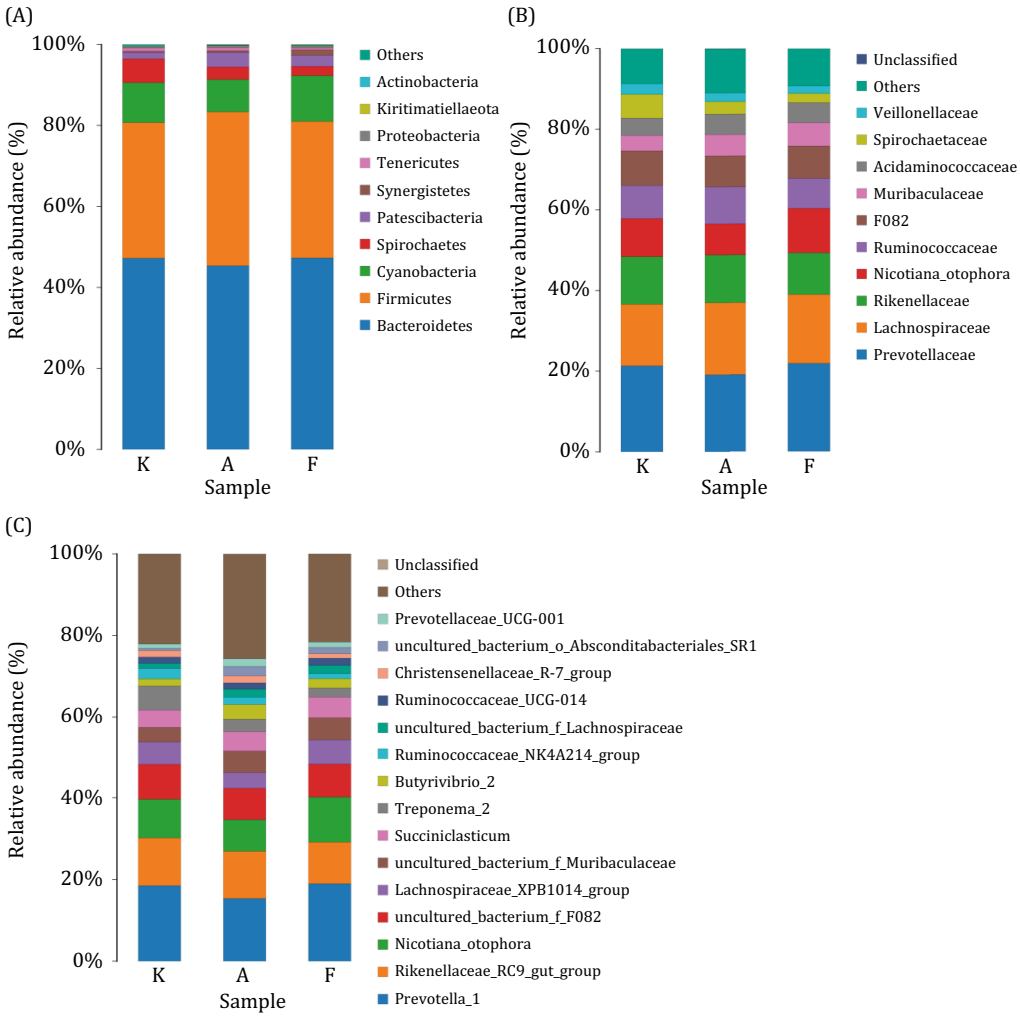
Figure 4 - Feasibility analysis of the sequencing data.

3.3. Bacterial community composition of RM at different taxonomic levels

Analysis of the bacterial composition and structure of RM among goats of different ages was conducted for different taxonomic levels (Figure 5). As indicated by the distribution results at the phylum level, Bacteroidetes were predominant in all samples, followed by Firmicutes (Figure 5A). A high abundance of Cyanobacteria was found in A4, F1, F2, F4, F6, K4, K5, and K7 samples, and high abundance of Patescibacteria was found in A1 and A6 samples. Interestingly, significant abundance of Synergistets was found only in A2, A5, F2, F3, F4, F6, and F7 (Figure 5A).

In addition to the phylum, bacterial community composition at family and genus levels was also analyzed. No groups displayed significant differences at the family level, and no significant differences were observed among the three groups (Figure 5B). Prevotellaceae, Lachnospiraceae, Rikenellaceae, Nicotiana-otophora, and Ruminococcaceae were the most abundant families in all samples. However, abundance of Spirochaetaceae in group K (except sample K4) was relatively higher than that in groups A and F.

At genus level, a total of 130 genera were identified from the 21 samples. The predominant genera included Prevotella_1, Rikenellaceae_RC9_gut_group, Nicotiana_otophora, and Uncultured_bacterium_f_F082 in all samples. The abundance of Treponema_2 in group K was relatively higher than in groups A and F (Figure 5C). Additionally, a plant-oriented sequence (i.e., Niotiana otophora) was found, although it did not differ among the groups, which needs special attention.



Groups: K - kids; F - fattening goats; A - adult goats.
Each bar represents the relative abundance of each bacterial taxon.
Taxa distributions at phylum (A), family (B), and genus (C) level.

Figure 5 - Composition of bacterial communities in rumen.

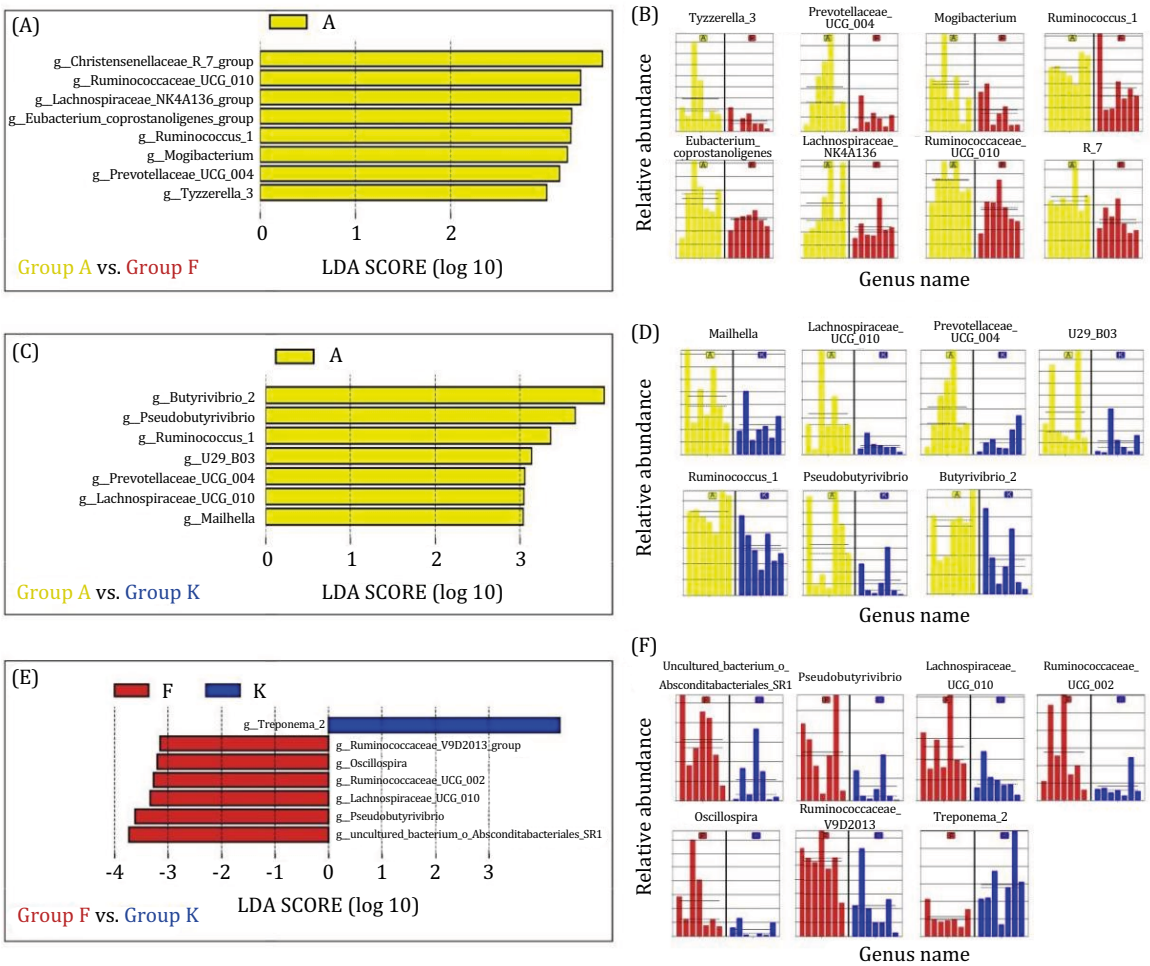
Overall, at the family and genus levels, differences in diversity and abundance among the three groups were gradually found, which was consistent with the previous analysis (Figure 3).

3.4. Correction analysis of RM between growth performances and rumen fermentation parameters

Relationships of RM and growth performance and rumen fermentation parameters was conducted by Pearson's analysis at genus level. Interestingly, none of genera was correlated with rumen fermentation parameters, while two genera were correlated with each of growth performance. For uncultured_bacterium_f_F082, it was strongly correlated with length of the goats ($r = 0.37$, $P<0.05$). Another genus, uncultured_bacterium_o_Absconditabacteriales_SR1, was negatively corrected with body weight ($r = -0.43$, $P<0.05$).

3.5. Discovery of age-related RM

We focused on the significant differences in the abundance of RM at different age developmental stages in goats. The OTU of each group were compared to identify the significant bacterial taxa against age differences. Linear discriminant analysis coupled with effect size (LEfSe) at the genus level was conducted to discover the bio-markers in this study. In RM communities (3.0 threshold value), marked differences were observed in eight taxa in group A when compared with group F (Figures 6A and 6B). In addition, differences in seven taxa were found between groups A and K



Groups: K - kids; F - fattening goats; A - adult goats.
Significant differences of bacterial taxa between groups A and F (A, B), between A and K (C, D), and between F and K (E, F).
Only scores that reached the linear discriminant analysis threshold >3.0 are displayed.

Figure 6 - Differences in groups investigated by using linear discriminant analysis coupled with effect size (LEfSe) at the genus level.

(Figures 6C and 6D). Furthermore, there were differences in six taxa in group F and one taxon in group K (Figures 6E and 6F).

The results (Figure 6) describe the difference of RM among goats of various ages, elucidating the association between age and RM. Christensenellaceae_R_7_group, Ruminococcaceae_UCG_010, Lachnospiraceae_NK4A136_group, Eubacterium_coprostanoligenes_group, Ruminococcus_1, Mogibacterium, Prevotellaceae_UCG_004, and Tyzzerella_3 were determined to have a higher abundance in group A (12-month-old goats) than in group F (six-month-old goats). Similarly, Butyrivibrio_2, abundance of Pseudobutyrivibrio, Ruminococcus_1, U29_B03, Prevotellaceae_UCG_004, Lachnospiraceae_UCG_010, and Mailhella were significantly higher in group A than in group K (one-month-old goats). Between groups K and F, the relative abundance of Treponema_2 was higher in group K, whereas the abundance of Ruminococcaceae_V9D2013_group, Oscillospira, Ruminococcaceae_UCG_002, Lachnospiraceae_UCG_010, Pseudobutyrivibrio, and Uncultured_bacterium_o_Absconditabacteriales_SR1 was lower in group K.

4. Discussion

Though recent studies on mammalian gut microbiota have covered many aspects, few of them have focused on the difference of the RM in goats, and especially at different ages of goats. In this study, the diversity and abundance of the RM of Guizhou White goats of various ages were analyzed. Results revealed that the abundance and diversity of RM in adult goats (12 months, group A) were higher than those in fattening goats (six months, group F) and kids (one month, group K).

Volatile fatty acids that are products of the fermentation of diets are shown to be essential to the rumen papillae development and nutrient source for host requirements (Suarez-Mena et al., 2016). In ruminants, VFA produced in the rumen meets 70-80% of the energy requirements for the epithelia and 50-70% of the energy requirements for the body (Shen et al., 2017). In our study, compared with group A, VFA concentrations (total VFA, propionate, and butyrate) were higher in group K and F. Acetate, valerate, and isovalerate in group K were lower than in the other two groups. Other studies have also revealed that early starter diet and alfalfa intake facilitate rumen development and change the pattern of ruminal fermentation (Wang et al., 2016). Feeding and management were crucial to ruminal fermentation. In our study, semi-intensive grazing could lead goat exposure to dietary fiber earlier, which can easier change the ruminal fermentation pattern at early ages.

Age has been regarded as a vital factor affecting animals' RM. Recently, a goat intestinal microbiota analysis revealed that there was no obvious difference between adult goats and kids (Wang et al., 2018). Another study on the rumen showed that the microbiome from fetus to adulthood exhibited dynamic fluctuations in goats (Zou et al., 2020). Furthermore, a gut microbiota study in goat kids from birth to post-weaning also showed differences in the microbiome (Zhuang et al., 2020). However, the current study found limited differences of rumen microbial abundance and diversity among goats of various ages (from 1 to 12 months), which is inconsistent with previous rumen-based reports on goats (Zou et al., 2020). Notably, although the differences in diversity of RM in different age groups were insignificant, the abundance of some bacterial taxa changed. Compared with that in group F, the proportion of eight taxa in group A increased. Seven different taxa were observed between group A and group K; differing ratios of six taxa in group F and one taxon in group K were also found. These changes in proportion may be the consequence of the host's development toward an improved structure during the course of age development.

This study confirmed that rumen fermentation parameters including VFA, especially acetate, propionate, and butyrate was different among the goats at different ages. However, none of VFA-related genera was found by Pearson's analysis, which was inconsistent with previous rumen-based reports on goats (Lv et al., 2019). Both length-related genus (uncultured_bacterium_f_F082) and body weight-related genus (Absconditabacteriales_SR1) were found in this study. These results advance the field of rumen microbial microbiology in animal performance connection.

The goats used in this study were able to roam freely on grassland in mountainous areas (same areas for all goats), the diet was also supplied with the same concentrated feed. Additionally, previous reports showed that the gut microbiota of goats might reach a stable state at an earlier age, and displayed similar distribution patterns in gut microflora during age development (Wang et al., 2018).

Our study has some limitations. Microorganisms were obtained from rumen preparations, and DNA extractions were inevitably present, even though we removed many of them. Different DNA extraction methods can affect the apparent structure of sheep rumen microbial communities (Henderson et al., 2013). To ensure the relative accuracy of the results, the equal DNA extraction of ruminal DNA from each animal were conducted in our study. The discovery of a plant-oriented sequence suggests that the non-microbial DNA such as plastogene from food sources needs to be removed.

Previous studies showed that different sampling techniques (the liquid and solid-associated fractions of the rumen) could cover different community structure in the rumen microbiome (Henderson et al., 2013; Cunha et al., 2011). In this study, we focused on discovering the rumen microorganism in the liquid-associated fractions. Analysis of the rumen content (both solid and liquid) was valuable and will be done in our future work.

In addition, we had a very low sample size (n = 7) in our study. It is worth noting that free access to natural grass feed may affect the RM in goats. Actually, it seems more likely that the uncontrolled feeding strategy could have contributed to the observed little microbial community differences, and that more controlled feeding could reduce this variation to yield communities that were even more similar.

5. Conclusions

Our results showed that more volatile fatty acids (i.e., acetate, propionate, and butyrate) were found in the six- and one-month-old goats. Compared with 12-month-old goats, eight and seven taxa decreased in six-month-old and one-month-old goats, respectively, compared with that in 12-month-old goats. In addition, seven taxa altered in six-month-old goats and one-month-old goats. Our study highlights the signature microbiota involved in animal health and production during age development and also provides a guiding tool for evaluating rumen health of ruminants.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: W. Zhou and H. Chen. Data curation: W. Zhou, C. Su and L. Xu. Formal analysis: W. Zhou, X. Wu, C. Su, L. Li, L. Xu, R.W. Akhtar and S.A.H. Shah. Funding acquisition: W. Zhou and H. Chen. Methodology: X. Wu, L. Li and R.W. Akhtar. Project administration: H. Chen. Resources: H. Chen. Supervision: H. Chen. Visualization: W. Zhou. Writing-original draft: W. Zhou, R.W. Akhtar, S.A.H. Shah and H. Chen. Writing-review & editing: W. Zhou and H. Chen.

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